SPECIFICATION

GENE CODING FOR A PROTEIN HAVING GLYCOSIDE TRANSFER ACTIVITY

Technical Field

The present invention relates to a gene coding for a protein having activity that transfers a glycoside to the 5 position of a flavonoid, and a process utilizing that gene.

Background Art

The flower industry strives to develop various new varieties. Changing the color of a flower is one way of effectively breeding a new variety. A wide range of colors have been successfully produced for nearly all commercial varieties using classical breeding methods. With these methods, however, since there are restrictions on the gene pool for each species, it is rare for a single species to have a broad range of colored varieties.

Flower colors are based on two types of pigments, namely flavonoids and carotinoids. Flavonoids contribute to color tones ranging from yellow to red and blue, while carotinoids contribute to color tones of orange or yellow. Flavonoid molecules that primarily contribute to flower color are anthocyanins which are glycosides of cyanidin, delphinidin, petunidin, peonidin, malvidin and pelargonidin, and different anthocyans cause remarkable changes in flower color. Moreover, flower color is also affected by auxiliary coloring by colorless flavonoids, metal complex formation, glucosylation, acylation, methylation and vacuolar pH (Forkmann, Plant Breeding, 106, 1, 1991).

The biosynthesis route of anthocyanins, which begins with phenylalanine, has been well understood (e.g., Plant Cell, 7, 1071-1083, 1995), and nearly all genes involved in the biosynthesis have been cloned. For example, among those genes thought to be involved in biosynthesis of

malonylshisonin (3-O-(6-O-(p-cumaloyl)-β-D-glucosyl)-5-O-(6-O-malonyl-β-D-glucosyl)-cyanidin), which is an anthocyanin of Perilla, those genes for which homologues have not yet been reported are only the flavonoid-3'-hydroxylase, UDP-glucose:anthocyanin (flavonoid) 5-O-glucosyl transferase (abbreviated as 5GT) and malonyl group transferase genes.

Among these, flavonoid-3'-hydroxylase is known to belong to the cytochrome P450 gene family (Plant Cell, 7, 1071-1083, 1995), and cytochrome P450 genes are surmised to demonstrate structural homology.

The hydroxyl group at the 3 position of flavonoid molecules is typically modified by glucose, and generally glucosylation and other modifications by glycoside are considered to increase the stability and solubility of anthocyanins (The Flavonoids, Chapman & Hall, 1994).

Genes coding for the UDP-glucose:anthocyanidin or flavonoid-3-glucosyl transferase (abbreviated as 3GT) that catalyze this reaction are obtained from numerous plants such as corn, barley, snapdragons and gentians, and their amino acid sequences mutually demonstrate significant homology. For example, the homology between the 3GT amino acid sequences of monocotyledonous corn and dicotyledoneous gentian is 32%, that between the 3GT amino acid sequences of monocotyledonous corn and monocotyledonous barley is 73%, and that between the 3GT amino acid sequences of dicotyledonous gentian and dicotyledonous eggplant is 46%.

In addition, the gene coding for UDP-ramnose:anthocyanidin 3-glucosidoramnosyl transferase (3RT) of petunias has also been cloned.

However, even though the hydroxyl group at the 5 position of the flavonoids of numerous plants is glucosylated, a gene for the enzyme (5GT) that catalyzes this reaction has yet to be obtained.

In addition, although there are examples of measuring the reaction by which glycoside is transferred to the 5

position of petunia and stock anthocyanins (Planta, 160, 341-347, 1984, Planta, 168, 586-591, 1986), these reports only describe the investigation of enzymological properties using crude extracts or partially purified products of flower petals, and there are no examples of this enzyme being purified to its pure form. In addition, since glycosyltransferases are typically biochemically unstable, enzyme purification is difficult.

Although there are hardly any cases in which color tone is changed by addition of glycoside to a flavonoid molecule, since aromatic acyl groups that have a significant effect on color tone are linked to a glucose molecule or ramnose molecule within an anthocyanin, regulation of the glycoside transfer reaction is important in terms of controlling anthocyanin biosynthesis, and ultimately in controlling flower color. Furthermore, as an example of changing flower color by regulating the expression of glycosyltransferase gene, the reaction by petunia 3RT has been controlled in transformed petunia to modify flower color.

Plant species, which can be transformed with a foreign gene, include, for example, roses, chrysanthemums, carnations, daisies, petunias, torenia, bellflowers, calanchoes, tulips and gladiolas.

Disclosure of the Invention

The inventors of the present invention therefore sought to obtain a gene that codes for a protein having activity that transfers a glycoside to the 5 position of a flavonoid, thereby leading to completion of the present invention.

For example, the 5 position hydroxyl group of the anthocyanins of chrysanthemums and some of the anthocyanins of roses and carnations are not glucosylated. The anthocyanin structure can be changed by introducing the 5GT gene obtained by the present invention into these plants.

In addition, although it is possible to change flower color and stabilize flavonoids by acylating flavonoids using the acyl group transferase gene described in International Publication No. WO96/25500, since the acyl group does not bond directly with the flavonoid, but rather bonds by way of a sugar, simply introducing an acyl group transferase gene alone is not sufficient for changing flower color and may even cause the flavonoid not to become stable.

However, by introducing the 5GT gene in combination with an acyl group transferase gene, sugar is bounded to the 5 position of the flavonoid thereby further allowing the flavonoid to be acylated. This can be expected to change the anthocyanin structure and cause the flower color to become bluish.

In addition, if expression of 5GT gene of a plant in which the 5 position of anthocyanin is glucosylated is suppressed with the antisense method or co-suppression method and so forth, transfer of glucose residue to 5 position can be inhibited. So that, flower color can be changed. For example, suppressing 5GT activity in gentian or bellflower can be expected to cause flower color to become reddish.

The inventors of the present invention isolated cDNA of 5GT from Perilla, torenia, verbena and petunia plants using gene recombination technology, and determined the nucleotide sequence of the structural gene. Namely, the inventors of the present invention provide a DNA sequence that codes for 5GT present in the tissue that expresses anthocyanins in these plants. Moreover, since this enzyme transfers glycoside to the 5 position of anthocyanin pigment, it can be used to change flower color and increase anthocyanin stability.

Embodiment for Carrying Out the Invention

The method of differential displacement, for example, can be used to obtain DNA that codes for the enzyme of the

present invention. In Perilla (Perilla frutescens), for example, there are varieties that accumulate anthocyanins (e.g., red forma) and those that do not (e.g., green forma). By cloning DNA present in varieties that accumulate anthocyanins but not present in varieties that do not, it is possible to obtain the DNA that codes for the enzyme of the present invention.

More specifically, RNA is extracted from the leaves of red forma and green forma, and cDNA is synthesized in accordance with standard methods. This is then separated by electrophoresis to isolate cDNA present in the cDNA library of red forma but not present in the cDNA library of green forma. Next, the red forma cDNA library is screened using the resulting cDNA as a probe to obtain the cDNA that codes for the enzyme of the present invention.

Once cDNA that codes for the enzyme of the present invention is obtained in the manner described above, this cDNA or its fragment is used as a probe to screening the cDNA libraries of other plants. As a result, the DNA that codes for the enzyme of the present invention can be obtained from those plants.

As an example of the screening, in the present invention, the DNA coding for the enzyme of the present invention is cloned from Perilla by the differential display method (Example 1). Next, DNA that codes for the enzyme of the present invention is obtained from verbena by screening of cDNAs from verbena (Verbena hybrida) using the cloned DNA of Example 1 as a probe (Example 2). Moreover, DNA coding for the enzyme of the present invention is obtained from torenia in the same manner (Example 3).

Then, it was confirmed that the proteins encoded in these DNAs have the enzymatic activity of the present invention.

Moreover, the DNA coding for the enzyme of the present invention was obtained from petunia (Example 4).

Examples of the DNAs of the present invention include

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that which codes for the amino acid sequence described in any one of SEQ ID NOs: 7 through 10 or 12. However, proteins having an amino acid sequence modified by addition and/or deletion of one or more amino acids and/or substitutions by one or more other amino acids are also known to maintain enzymatic activity similar to the original protein. Thus, genes coding for a protein that has an amino acid sequence modified by addition and/or deletions of one or more amino acids and/or substitutions by one or more other amino acids relative to the amino acid sequence described in any one of SEQ ID NOs: 7 through 10 or 12, and still maintains activity of transferring a glycoside to the 5 position of a flavonoid, also belong to the present invention.

The present invention also relates to a gene coding for a protein which gene hybridizes to a nucleotide sequence described in any one of SEQ ID NOs: 1 through 4 or 6, or to a nucleotide sequence that codes for an amino acid sequence described therein or to their portions, for example a portion coding for at least six amino acids of a consensus region, under conditions of 2 to 5 x SSC, and for example, 5 x SSC, and 50°C, and that has activity of transferring a glycoside to the 5 position of a flavonoid. Furthermore, the optimum hybridization temperature varies according to the nucleotide sequence and its length, and it is preferable that the hybridization temperature be lower the shorter the nucleotide sequence. For example, a temperature of 50°C or lower is preferable in the case of a nucleotide sequence (18 bases) coding for six amino acids.

Although examples of genes selected by hybridization in this manner include those which are naturally-occurring such as those derived from plants, examples of which include a gene derived from verbena and torenia, they may also be those derived from other plants, examples of which include petunias, roses, carnations and hyacinths. In addition, genes selected by hybridization may also be cDNA or genomic DNA.

Moreover, the present invention also relates to a gene coding for a protein having an amino acid sequence having homology of 30% or more, preferably 50% or more, for example 60% or 70% or more, and in some cases, 90% or more relative to an amino acid sequence of any of SEQ ID NOs: 7 through 10 or 12, and having activity that transfers a glycoside to the 5 position of a flavonoid. Namely, as indicated in Examples, DNA coding for the enzyme of the present invention demonstrates homology of 20 to 30% in comparison with other glycosyltransferase genes. Thus, the present invention includes genes coding for a protein that having homology of 30% or more with an amino acid sequence described in any one of SEQ ID NOs: 7 through 10 or 12, and has glycosyltransferase activity.

In addition, as is clear from a comparison of the results of Examples 1 through 4, the amino acid sequence of the enzyme of the present invention varies according to the species, with interspecies homology being 50% or more (see Examples 3 and 4), and for example 60 to 70% (see Example 2), while the homology of the amino acid sequences of the enzymes derived from the same species is 90% or more (see Example 1). Thus, genes coding for a protein that has an amino acid sequence having homology of 50% or more, for example 60-70% or more, and in some cases, 90% or more, relative to an amino acid sequence described in any one of SEQ ID NOs: 7 through 10 or 12, and maintains the glycosyltransferase activity of the present invention are included in the present invention.

As is described in detail in Examples, DNA having a native nucleotide sequence is obtained by, for example, screening of a cDNA library.

In addition, DNA coding for an enzyme having a modified amino acid sequence can be synthesized using ordinary site-specific mutagenesis and PCR based on the nucleotide sequence of a native DNA. For example, a DNA fragment containing a site at which a modification is desired to be introduced is obtained by restriction enzyme

digestion of cDNA or genomic DNA obtained as described above. By using this as a template, site-specific mutagenesis or PCR is performed using a primer containing the desired mutation to obtain a DNA fragment containing the desired modification. This is then ligated to DNA coding for another portion of the target enzyme.

Alternatively, in order to obtain DNA coding for an enzyme having a shortened amino acid sequence, for example, DNA coding for an amino acid sequence that is longer than the target amino acid sequence, for example that coding for the entire amino acid sequence, is digested by a desired restriction enzyme, and in the case the resulting DNA fragment does not code for the entire target amino acid sequence, the deficient portion should be supplemented by ligating synthetic DNA.

In addition, by expressing this clone using a gene expression system in E. coli or yeast and measuring enzyme activity, the resulting gene can be confirmed to code for glycosyltransferase, and by clarifying the translation region of glycosyltransferase gene that transfers glycoside to the 5 position of a flavonoid, a gene is obtained that codes for the glycosyltransferase claimed in the present invention. Moreover, by expressing said gene, the target transferase protein that transfers a glycoside to the 5 position of a flavonoid can be obtained.

Alternatively, the protein can be obtained by using antibody to an amino acid sequence described in any one of SEQ ID NOs: 7 through 10 or 12.

Thus, the present invention also relates to a recombinant vector containing the above-mentioned DNA, and more particularly, to an expression vector and a host transformed with the vector. Both prokaryotes and eukaryotes can be used for the host. Examples of prokaryotes that can be routinely used for the host include bacteria, for example, the genus Escherichia such as Escherichia coli, and the genus Bacillus such as Bacillus subtilis.

Examples of eukaryotes that can be used include lower eukaryotes such as eucaryotic microorganisms including fungi such as yeast or mold. Examples of yeast includes the genus Saccharomyces such as Saccharomyces cerevisiae, while examples of molds include the genus Aspergillus such as Aspergillus oryzae and Aspergillus niger, as well as the genus Penicillium. Moreover, animal or plant cells can also be used, examples of animal cells including mouse, hamster, monkey and human cell systems. Moreover, insect cells such as silkworm cells or adult silkworms themselves can be used as hosts.

The expression vectors of the present invention contain an expression control region, such as a promoter, terminator or an origin of replication, depending on the type of host in which they are to be introduced. Examples of promoters of bacterial expression vectors include conventionally used promoters such as trc promoter, tac promoter and lac promoter, while examples of yeast promoters include glyceroaldehyde triphosphate dehydrogenase promoter and PH05 promoter. Examples of mold promoters include amylase and trpC. In addition, examples of promoters for animal cell hosts include viral promoters such as SV40 early promoter and SV40 late promoter.

Preparation of expression vector can be performed in accordance with standard methods using restriction enzyme, ligase and so forth. In addition, transformation of a host by an expression vector can also be performed in accordance with standard methods.

In the process for producing the above-mentioned protein, a host transformed with the expression vector is cultured, cultivated or bred, the target protein can be recovered and purified from the resulting culture in accordance with standard methods, examples of which include filtration, centrifugation, cell homogenation, gel filtration chromatography and ion exchange chromatography.

Furthermore, although the present specification

describes transferases derived from Perilla, verbena, torenia and petunia wherein the transferases that transfer glycoside to the 5 position of a flavonoid (which may be simply referred to as "glycosyltransferase" in the present invention), a gene that codes for said enzyme can be cloned, by entirely or partially altering the purification method of said enzyme so as to purify a glycosyltransferase of another plant, and determining the amino acid sequence of said enzyme. Moreover, by using cDNA of the glycosyltransferase derived from Perilla of the present invention as a probe, cDNA of a different glycosyltransferase was able to be obtained from Perilla, and cDNA of a different glycosyltransferase was able to be obtained from a different plant. Thus, other glycosyltransferase genes can be obtained by using a portion or the entirety of a glycosyltransferase gene.

In addition, as indicated in the present specification, by purifying glycosyltransferase from Perilla, verbena, torenia and petunia to obtain antibody to said enzyme in accordance with standard methods, cDNA or chromosomal DNA produces protein which reacts with that antibody that can be cloned. Thus, the present invention is not limited to only genes of glycosyltransferases derived from Perilla, verbena, torenia and petunia, but also relates to glycosyltransferase in the broad sense.

Moreover, the present invention also relates to a plant, its progeny or their tissue for which color has been adjusted by introduction of glycosyltransferase gene, and their form may be that of cut flowers as well.

In addition, UDP-glucose is an example of a glycoside donor in the glycoside transfer reaction of glycoside that include anthocyanin in the present specification.

Examples

The following provides a detailed explanation of the present invention based on Examples. Unless specified otherwise, the experimental procedure was performed in

accordance with the methods described in Molecular Cloning (Cold Spring Harbor, 1989), New Biochemistry Experimental Manual (Kagaku Dojin, 1996) and International Patent Laid-Open Publication No. WO96/25500.

Example 1 Cloning of a Gene Specifically Expressed in Red Forma

(1) Differential Display

Perilla (Perilla frutescens) includes varieties that accumulate anthocyanins in their leaves (for example, red forma (Sakata-no-tane)), and varieties that do not accumulate anthocyanins (for example, blue forma (Sakata-no-tane)). The structure of the major anthocyanin is reported to be malonylshisonin (3-0-(6-0-(p-cumaloyl)-β-D-glucosyl)-5-0-(6-0-malonyl-β-D-glucosyl)-cyanidin) (Agri. Biol. Chem., 53:197-198, 1989).

Differential display is a method reported in Science, 257, 967-971 (1992), and is used, for example, to obtain genes that are expressed tissue-specifically.

Total RNA was extracted from the leaves of the above-mentioned two types of Perilla by the hot phenol method (Plant Molecular Biology Manual, Kluwer Academic Publishers, 1994, pp. D5/1-13). Poly A + RNA was purified from the resulting total RNA using an mRNA separator kit (Clonetech). 0.9 μg of poly A + RNA were reverse-transcribed in 33 μl of reaction mixture using oligo-dT primer added an anchor (GenHunter, H-T11G, H-T11A and H-T11C) to obtain single strand cDNA. Using this cDNA as a template, PCR was performed using the same oligo-dT primer added an anchor and synthetic primers (GenHunter, H-AP1 through 8) as primers.

The volume of the PCR reaction mixture was 20 μl, and it contained 2 μl of cDNA solution, 0.2 μM of any one of H-T11G, H-T11A or H-T11C primer, 0.2 μM of any primer from H-AP1 through H-AP8, 0.12 μM dNTP, 5 or 10 μCi of [^{32}P]dCTP, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.01% Triton X-100, 1.25 mM $MgCl_2$ and 1 unit of Taq polymerase. The reaction conditions comprised holding the temperature at

72°C for 20 seconds followed by repeating the reaction for 40 cycles with one cycle comprising raising the temperature to 94°C for 30 seconds, lowering to 40°C for 2 minutes and raising to 72°C for 30 seconds, and then holding the temperature at 72°C for 5 minutes.

The DNA fragments amplified in this manner were separated by the same polyacrylamide gel electrophoresis as used for DNA Sequencing. After drying the gel, the gel was exposed to X-ray film. Among the resulting approximately 2,600 bands, there were 36 bands observed only in the red forma as a result of comparing the two varieties. They were cut out of the dried gel and eluted into 100 µl of water. The eluted DNA was precipitated with ethanol and dissolved in 20 µl of water. Using a half amount of each DNA as a template, the PCR reaction was performed as described above, and amplified fragments were obtained for 33 of DNA fragments. Library screening and northern analysis were then performed using these DNA fragments.

(2) Northern Analysis

Northern analysis was performed according to the method described below using the above 33 types of DNA probes. After separating poly A + RNA derived from red forma and green forma with formamide gel containing 1.2% agarose, the poly A + RNA was transferred to a Nylon membrane. This membrane was hybridized with the above-mentioned DNA probes labeled with [^{32}P] for overnight at 65°C in the presence of 5XSSPE, 5X Denhalt's solution, 0.5% SDS and 20 µg/ml of denatured salmon sperm DNA. The hybridized membrane was washed at 65°C in 1XSSPE and 0.1% SDS solution and subjected to autoradiography. As a result, only five probes were specifically expressed in red forma. These clones are predicted to be genes involved in the biosynthesis of anthocyanins.

(3) Screening of cDNA Library

A cDNA library with λgt10 as a vector was prepared using the poly A + RNA obtained from the leaves of red

forma and the Complete Rapid Cloning System λgt10 (Amersham). This cDNA library was screened with the five DNA fragments described above to obtain cDNA corresponding to each fragment. Among these, a clone named 3R5 was obtained using a DNA fragment obtained by H-T11A and H-AP3 primers, and this clone demonstrated homology of approximately 26% at the amino acid level with previously reported corn flavonoid-3-0-glucosyl transferase.

In addition, clones designated as 3R4 and 3R6 were obtained by library screening using the same probes, and these demonstrated an extremely high level of homology with 3R5. The complete nucleotide sequences and deduced amino acid sequences of 3R4 and 3R6 are shown in SEQ ID NO: 1 and SEQ ID NO: 2 of the Sequence Listing, respectively. In addition, the deduced amino acid sequences of the proteins encoded by 3R4 and 3R6 demonstrated homology of 92%.

A clone designated as 8R6 was obtained using a DNA fragment obtained by H-T11G and H-AP8 primers, and this clone did not demonstrate significant homology with any sequences reported so far. This sequence is shown in SEQ ID NO: 5 of the Sequence Listing. Although there is a strong possibility that 8R6 is a gene involved in the biosynthesis of anthocyanins, since its structure lacks homology with genes reported so far, it is predicted to be a new gene involved in anthocyanin biosynthesis.

In consideration of the anthocyanin structure in Perilla (the previously mentioned malonylshisonin), it is predicted that this gene is a malonyl transferase. In order to verify this, this gene should be expressed in yeast and E. coli followed by reacting with anthocyanin and malonyl-CoA as substrates. Such an experiment can be carried out using, for example, the method described in International Publication No. WO96/25500. Malonyl transferase gene is useful in terms of artificially altering anthocyanin structure.

(4) Expression of 3R4 cDNA in Yeast

An approximately 1.5 kb DNA fragment obtained by blunting the BstXI cleavaged site of p3R4 using T4 DNA polymerase (Takara Shuzo) and then cutting out at the BamHI cleavage site in the adapter, and an approximately 8 kb DNA fragment obtained by blunting the EcoRI cleaved end of pYE22m and then digesting with BamHI were ligated to obtain a plasmid that was designated as pY3R4.

Furthermore, E. coli strain JM109 having pYE22m was named Escherichia coli SBM335, and deposited at the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology as FERM BP-5435. In pY3R4, cDNA coding for glycosyltransferase has been ligated downstream of the promoter for glyceroaldehyde triphosphate dehydrogenase lone of the constitutive yeast promoter, and transcription is controlled by this promoter.

Using pY3R4, yeast Saccharomyces cerevisiae G1315 (Ashikari, et al., Appl. Microbiol. Biotechnol., 30, 515-520, 1989) was transformed according to the method of Ito, et al. (Ito, et al., J. Bacteriol., 153, 163-168, 1983). The transformed yeast was selected according to recovery of tryptophan synthesis ability. The resulting transformed strain was cultured for 24 hours at 30°C with shaking in 10 ml of Burkholder's medium (Burkholder, Amer. J. Bot., 30, 206-210) containing 1% casamino acids.

In order to conduct a control experiment, yeast that spontaneously recovered tryptophan synthesis ability was also cultured in the same manner. After collecting the yeast, the cells were suspended in suspension buffer (100 mM phosphate buffer (pH 8.5), 0.1% (v/v) 2-mercaptoethanol, 10 μM APMSF and 100 μM UDP-glucose) followed by the addition of glass beads (Glass Beads, 425-600 microns Acid-Wash, Sigma) and vigorous shaking to crush the cells. The crushed cells were then centrifuged for 20 minutes at 15,000 rpm and the supernatant was used as a crude enzyme solution for the measurement of enzyme activity described below.

(5) Measurement of Enzymatic Activity

After allowing 50 µl of reaction mixture containing 20 µl of crude enzyme solution (100 mM phosphate buffer (pH 8.5), 670 µM cyanidin-3-glucoside, 1 mM UDP-glucose) for 10 minutes at 30 °C, 50 µl of 50% acetonitrile solution containing 0.1% TFA was added to stop the reaction. Supernatant obtained by centrifuging for 5 minutes at 15,000 rpm was passed through a Samprep LCR4(T)-LC filter (Millipore) so as to remove impurities. This was then analyzed by high-performance liquid chromatography (HPLC). Analysis was performed using a reverse phase column (Asahipak ODP-50, 4.6 mm diameter x 250 mm, Showa Denko), the mobile phase consisted of 0.5% TFA/H_2O for solution A and 0.5% TFA 50% CH_3CN for solution B. The flow rate was 0.6 ml/min. and the fractions were eluted at a gradient of B20% → B100% (20 min) followed by holding at B100% for 5 minutes.

20 µl of reaction mixture was used for analysis. A520 nm, AUFS 0.5 (Shimadzu SPD-10A) and a photodiode array detector (Shimadzu SPD-M6A) at an absorbance of 600-250 nm were used for detection. In the case of reaction of yeast crude enzyme solution that expressed pY3R4, in addition to the substrate cyanidin-3-glucoside (retention time: 17 minutes), a new peak was observed at retention time of 14.5 minutes. Since it was not observed in the case of reaction of yeast crude enzyme solution of the control experiment, this new peak was considered to be generated due to the activity of protein originated from pY3R4. As a result of co-chromatography with cyanidin-3,5-diglucoside, the retention time of this peak coincided with that of cyanidin-3,5-diglucoside, and their absorption spectra were also identical to each other. Based on these observations, 3R4 cDNA of Perilla was found to code for 5GT.

Example 2 Cloning of 5GT Gene of Verbena hybrida

(1) Preparation of cDNA Library

Petals were collected from Verbena variety Hanatemari

violet (Suntory) and ground by a mortar and pestle in liquid nitrogen. RNA was extracted from the ground tissues according to a method using guanidine thiocyanate/cesium chloride, and poly A + RNA was obtained by the method recommended by the manufacturer using Oligotex (Takara Shuzo). The method using guanidine thiocyanate/cesium chloride was carried out in accordance with the method described in detail in Methods in Molecular Biology, Vol. 2 (Humana Press Inc., 1984) by R. McGookin and Robert J. Slater, et al.

Using the resulting poly A + RNA as a template, double-stranded cDNA was synthesized using the ZAP-cDNA synthesis kit (Stratagene), then, a cDNA library was prepared using the Uni-ZAP XR Cloning Kit (Stratagene) according to the method recommended by the manufacturer.

(2) Cloning of 5GT cDNA

The λ phage library obtained as described above was screened in the following manner using the p3R4 cDNA of Perilla as a probe. The filters were maintained at 42°C for 1 hour in hybridization buffer (5X SSC, 30% formamide, 50 mM sodium phosphate buffer (pH 7.0), 3% SDS 2% blocking reagent (Boehringer), 0.1% lauroylsarcosine, 80 μg/ml of salmon sperm DNA). DIG-labeled Perilla 5GT cDNA, p3R4 cDNA, fragment was added to the hybridization solution and the filters were incubated for further 16 hours.

After washing the filters with washing solution (5X SSC 50°C, 1% SDS), the positive clones labeled with anti-DIG-alkaline phosphate were immunologically detected using 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium salt according to the method described by the manufacturer (Boehringer).

As a result, seven positive clones were obtained. These cDNA were excised on plasmid pBluescript SK using the method recommended by Stratagene. When the lengths of the cDNA were investigated by agarose gel electrophoresis, insertion of a maximum length of 2.0 kb was observed.

(3) Determination of Nucleotide Sequence

Plasmids were extracted from the resulting clones, and the nucleotide sequences near the 3' and 5' ends of the cDNA were determined according to the dideoxy sequence method using fluorescent reagent as recommended by Perkin-Elmer with the ABI 373A sequencer (Perkin-Elmer). As a result, five of the seven clones had mutually same nucleotide sequences although the lengths of the cDNA were different. The entire nucleotide sequence of pSHGT8 was determined. Determination of nucleotide sequences was performed as described above by either using the Kilo-Sequence Deletion Kit (Takara Shuzo) to obtain a series of deleted cDNA clones, or by using an oligoprimer specific for the internal sequence of pSHGT8.

(4) Comparison of the Nucleotide Sequence and the Amino Acid Sequence

The cDNA inserted into pSHGT8 had the length of 2062 bp, and included an open reading frame (ORF) consisting of 1386 bp in length (including a stop codon). This sequence is shown in SEQ ID NO: 3. The amino acid sequence of this ORF had homology of 68% with the amino acid sequence of Perilla 5GT encoded by p3R4, and homology of 64% with that encoded by p3R6. In addition, it also had homology of 22 to 25% with the 3GTs of monocotyledonous and dicotyledoneous plants, and homology of 21% with petunia 3RT.

(5) Expression in Yeast and Measurement of Enzymatic Activity

An approximately 2.0 kb DNA fragment obtained by digesting pSHGT8 with BamHI/XhoI, and an approximately 8 kb DNA fragment obtained by digesting pYE22m with BamHI/SalI were ligated, and the resulting plasmid was designated as pYHGT8. pYHGT8 was expressed in yeast cells in the same manner as Example 1, and the enzymatic activity of the protein encoded by pSHGT8 was measured. As a result, in the reaction mixture containing the crude enzyme solution of yeast transformed with pYHGT8, a product was obtained that coincided with cyanidin-3,5-

diglucoside in both retention time and absorption spectrum. Based on this observation, the pSHGT8 cDNA of Verbena was determined to code for 5GT.

Example 3 Cloning of Trenia 5GT Gene

(1) Preparation of cDNA Library

Petals were collected from torenia variety Summer Wave Blue (Suntory) and ground in a mortar and pestle in liquid nitrogen. RNA was extracted from the ground tissues according to a method using guanidine thiocyanate/cesium chloride, and poly A + RNA was obtained by the method recommended by the manufacturer using Oligotex (Takara Shuzo). The method using guanidine thiocyanate/cesium chloride was carried out in accordance with the method described in detail in Methods in Molecular Biology, Vol. 2 (Humana Press Inc., 1984) by R. McGookin and Robert J. Slater, et al.

Using the resulting poly A + RNA as a template, double-strand cDNA was synthesized using the ZAP-cDNA synthesis kit of Strategene, then, a cDNA library was prepared using the Uni-ZAP XR Cloning Kit (Stratagene) according to the method recommended by the manufacturer.

(2) Cloning of 5GT cDNA

The λ phage library obtained as described above was screened in the same manner as Example 2 using the p3R4 cDNA of Perilla as a probe. As a result, eight positive clones were obtained. After excision of the cDNA on plasmid pBluescript SK, the lengths of the cDNA were investigated by agarose gel electrophoresis, which revealed that a maximum length of insertion was 1.6 kb.

(3) Determination of Nucleotide Sequence

Plasmids were extracted from the resulting clones, and the nucleotide sequences near both 5' and 3' ends were determined in the same manner as Example 2. As a result, six of the eight clones were considered to have mutually same nucleotide sequences although the lengths of the cDNA were different. The entire nucleotide sequence of pSTGT5 cDNA was determined.

(4) Comparison of the Nucleotide Sequence and the Amino Acid Sequence

The cDNA encoded in pSTGT5 was of 1671 bp in length, and included an open reading frame (ORF) consisting of 1437 bp in length (including a stop codon). This sequence is shown in SEQ ID NO: 4. The amino acid sequence of this ORF had homology of 58% with the amino acid sequence of Perilla 5GT encoded by p3R4, and homology of 57% with that encoded by p3R6, and, homology of 57% with that encoded by Verbena pSHGT8. In addition, it also had homology of 19 to 23% with the 3GT of monocotyledonous and dicotyledoneous plants, and homology of 20% with petunia 3RT.

(5) Expression of 5GT gene in Yeast

An approximately 1.6 kb DNA fragment obtained by digesting pSTGT5 with SmaI/KpnI, and an approximately 8 kb DNA fragment obtained by blunting the EcoRI-digested site of pYE22m and then digesting with KpnI were ligated, and the resulting plasmid was designated as pYTGT5. pYTGT5 was expressed in yeast cells in the same manner as Example 1, and the enzymatic activity of the protein encoded by pSTGT5 was measured. As a result, in the reaction mixture containing the crude enzyme solution of yeast transformed with pYTGT5, a product was obtained that coincided with cyanidin-3,5-diglucoside in both retention time and absorption spectrum. Based on this observation, the pSTGT5 cDNA of Torenia was determined to code for 5GT.

Example 4 Cloning of Petunia 5GT Gene

(1) Preparation of cDNA Library

A cDNA library was prepared by RNA extracted from petals of the Petunia variety Old Glory Blue in the manner described in detail by T. Holton, et al. (Plant Journal, 1993 4: 1003-1010)

(2) Cloning of 5GT cDNA

The cDNA library was screened in the same manner as Example 2 using the mixture of 5GT cDNAs of Perilla, torenia and verbena obtained in the manner described above

as probes. As a result, four positive cDNA clones were obtained and excised on plasmid pBluescript SK. The lengths of the cDNA were investigated by agarose gel electrophoresis, cDNA of a maximum length of 2.0 kb was observed.

(3) Determination of the Nucleotide Sequence

Plasmids were extracted from the resulting clones, and the nucleotide sequence near the 5' end was determined in the same manner as Example 2. As a result, two of the four clones, pSPGT1, were appeared to code an amino acid sequence with a high degree of homology with those of 5GT from Perilla, torenia and verbena obtained thus far. Therefore, the entire nucleotide sequence of pSPGT1 was determined.

(4) Comparison of the Nucleotide Sequence and the Amino Acid Sequence

The pSPGT1 cDNA was 2015 bp in length, and included an open reading frame (ORF) consisting of 1407 bp (including a stop codon). This sequence is shown in SEQ ID NO: 6. The amino acid sequence of this ORF had homology of 57% with that of 5GT encoded by p3R4 of Perilla, homology of 54% with that encoded by p3R6, 55% with that encoded by pSHGT8 of verbena, and 51% of that encoded by pTGT5 of torenia. In addition, it also had homology of 20 to 29% with the 3GT of monocotyledonous and dicotyledoneous plants, and homology of 20% with petunia 3RT. Based on this observation, pSPGT1 cDNA obtained from petunia is considered to code for 5GT.

Industrial Applicability

As has been described above, cDNA coding for enzymes that transfer a glycoside to the 5 position of a flavonoid originating in Perilla, verbena, torenia and petunia were cloned and their nucleotide sequences were determined. In addition, the isolated cDNAs were clearly shown to code for 5GT by the enzymatic activity of their protein expressed in yeast. Introducing of these cDNAs into a

suitable plant expression vector and transferring the resulting expression constructs into a plant makes it possible to provide, increase or decrease 5GT activity in the transformed plant, which leads to regulation of flower color. In addition, by using this enzyme, the structure of anthocyans can be altered or more stable anthocyans can be synthesized either in plants or in vitro.